Toxicity & Teratogenicity Studies in Ascorbic Acid-**Ascorbic Acid**-FDA Contract
#72-345
No Date

ASCORBIC ACID

TOXICITY and TERATOGENICITY STUDIES in ASCORBIC ACID

FDA CONTRACT #72-345

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STUDIES on the TOXICITY and TERATOGENICITY of ASCORBIC ACID

SUMMARY and CONCLUSIONS

Ascorbic acid (71-65) was found to be embryo-toxic under the conditions of these studies. The injection of this compound into the air cell or yolk resulted in statistically significant increases in embryo mortality.

Under the conditions of these studies ascorbic acid was not teratogenic at levels up to 200 mg/kg.

GENERAL PROCEDURES

The protocols as specified under FDA Contract #72-345 were followed in the investigation of toxicity and potential teratogenicity of the specified substance. The toxicity of the substance was evaluated from the percentage hatch of embryos injected either in the air cell or yolk at either zero hours (pre-incubation) or after 96 hours incubation to provide four separate evaluations.

EGG SOURCE AND HANDLING

All eggs used in these investigations were from Shaver Starcross pullets housed at the Poultry Research Center of the University of Arizona in Tucson. The parent stock was maintained on the University of Arizona breeder diet which had been formulated to provide more than adequate amounts of all the known nutrients required by the breeding hen.

The feed was specially prepared to assure no contaminations and did not contain any additive drugs such as antibiotics. All eggs prior to use (within 48 hours of lay) were candled to remove any containing blood spots, abnormal air cells or abnormal shells, and only clean eggs ranging in weight from 23 - 26 ounces per dozen were used.

The supply flock was tested to assure the absence of Pullorum and Mycoplasma gallisepticum.

The eggs were incubated in forced draft Jamesway 252 machines with automatic temperature and humidity controls and an automatic turning device.

COMPOUND HANDLING FOR INJECTION

The substance tested was solubilized in a number of the prescribed solvents in order to determine the maximum concentrations which could be employed. Where possible, water was the solvent of choice. Maximum

injection volume was 0.05 ml. and all solvents and glassware were autoclaved prior to preparation of the solutions for use. The dose levels were administered with a microliter syringe using sterilized needles.

The preliminary range-finding studies using each of the administration routes and times were carried out with 10 - 25 eggs per dose level and included solvent controls, untreated controls and either drilled or pierced controls.

The actual dose-response protocol was carried out in two or more injections on different days to produce a minimum of 100 eggs at each dose level in five or more levels selected from the range- finding studies.

EXAMINATIONS OF EMBRYOS AND CHICKS

Eggs were candled daily and the dead embryos removed, examined and any abnormalities recorded. Five chicks from each dose level in each hatch were X-rayed to determine any skeletal abnormalities. Additional eggs injected at the approximate LD-50 level and an additional level below that were incubated and embryos at 8, 14, 17 days and hatch chicks removed for histopathological examinations.

In additional studies representative chicks from the dose-response protocol were saved. These chicks were housed in electrically-heated battery brooders with raised wire floors and fed University of Arizona diets. Feed consumption and growth rates were evaluated at 6 weeks of age and a sample of the birds sacrificed for gross and histopathological examinations.

DATA HANDLING

All data were coded on forms provided by FDA for computer input. In addition to summaries of mortalities and abnormalities, a number of statistical evaluations were carried out. These statistical analyses included the following for both mortality and the incidence of abnormal embryos:

1. Chi-square tests for all dose levels and for each level against the solvent control.
2. Linear regression analyses + chi square test of linearity.
   a. % response against dose
   b. % response against log dose
   c. log % response against dose
   d. arcsin transformation against dose
   e. arcsin transformation against log dose
3. Log dose against Probit using Finney's maximum likelihood method.
   a. Where significant, the LD-30, 50, 70 and 90's were estimated with 95% confidence intervals.
4. One-way analyses of variance.
5. Linear regression with replication.

Ascorbic Acid (71-65) was solublized in water for the four test protocols. Maximum dose level of 200 mg/kg was obtained with 200 mg/ml.

MORTALITY

The mortality data obtained in the four test protocols are shown in Tables 1 - 4. When the air cell administration route was employed, ascorbic acid produced a significant increase in embryo mortality at levels of 40 mg/kg and above in the 96 hr series (Table 5). In the 0 hr series none of the individual dose levels were statistically significant in comparison with the water control groups; however, when all doses were compared to the controls a significant chi-square value was obtained ($P < 0.05$). This was also true with each of the injection times for yolk administration of ascorbic acid

Probit analyses yielded a significant linear relationship between log dose and probit of mortality for only the air cell-96 hr series (Table 6). These data provided an estimate of 57.7 mg/kg as the LD-50 dose under these conditions.

TERATOLOGY

The data obtained on abnormalities and H-S-V-L abnormalities are shown in Tables 1 - 4. Chi-square analyses of these data failed to indicate a significant increase in the occurrence of abnormal embryos as a result of ascorbic acid administration (Tables 7 & 9). Probit analyses of the data on abnormality incidence was also not statistically significant (Table 8). The individual teratological findings obtained with ascorbic acid administration in the four test protocols are shown in Table 10.

TABLE 1

ASCORBIC ACID
in WATER
AIR CELL - 0 HRS

e, m	No. Fertile	Mortality %	Mortality #	Abnormal Total %	Abnormal Total #	Abnormal H-S-V-L %	Abnormal H-S-V-L #	Abnormalities by category: Head %	Head #	Skeletal %	Skeletal #	Viscera %	Viscera #	Limbs %	Limbs #	Struc-tural %	Struc-tural #	Toxic Response %	Toxic Response #	Functional %	Functional #
80. 0	137	16.78	23	1.45	2	1.45	2	0.72	1			0.72	1			0.72	1				
40. 0	100	14.00	14	1.00	1	1.00	1	1.00	1												
20. 0	99	10.10	10	0.00	0	0.00	0														
10. 0	100	7.00	7	0.00	0	0.00	0														
5.0 0	100	8.00	8	0.00	0	0.00	0														
2.5 0	136	19.11	26	3.67	5	3.67	5	1.47	2					2.20	3			0.73	1		
drilled ed	99	12.12	12	0.00	0	0.00	0														
untreated ed	238	7.98	19	0.42	1	0.00	0													0.42	1

SUMMARY - ALL DOSE LEVELS

536	11.57 62	0.56 3	0.56 3	0.37 2		0.19 1		0.19 1			

TABLE 2

ASCORBIC ACID
in WATER
AIR CELL - 96 HRS

Dose, ppm	No. Fertile	Mortality %	Mortality #	Abnormal Total %	Abnormal Total #	Abnormal H-S-V-L %	Abnormal H-S-V-L #	Abnormalities by category: Head %	Head #	Skeletal %	Skeletal #	Viscera %	Viscera #	Limbs %	Limbs #	Struc-tural %	Struc-tural #	Toxic Response %	Toxic Response #	Functional %	Functional #
200.0	20	100.00	20	0.00	0	0.00	0														
160.0	20	100.00	20	0.00	0	0.00	0														
120.0	20	100.00	20	0.00	0	0.00	0														
80.0	119	71.42	85	2.52	3	1.68	2					0.84	1	0.84	1	1.68	2	0.84	1		
40.0	156	29.48	46	2.56	4	1.28	2	0.64	1					0.64	1	0.64	1	0.64	1	0.64	1
20.0	100	10.00	10	0.00	0	0.00	0														
12.0	99	8.08	8	2.02	2	2.02	2	2.02	2												
4.0	100	7.00	7	2.00	2	0.00	0									2.00	2				
0.0	157	6.36	10	0.00	0	0.00	0														
illed	120	3.33	4	0.83	1	0.00	0											0.83	1		
reated	238	7.98	19	0.42	1	0.00	0													0.42	1

SUMMARY - ALL DOSE LEVELS

634	34.07 216	1.74 11	0.95 6	0.47 3		0.16 1	0.32 2	0.79 5	0.32 2	0.16 1

TABLE 3

ASCORBIC ACID
in WATER
YOLK - 0 HRS

Dose, ppm	No. Fertile	Mortality %	Mortality #	Abnormal Total %	Abnormal Total #	Abnormal H-S-V-L %	Abnormal H-S-V-L #	Abnormalities by category: Head %	Head #	Skeletal %	Skeletal #	Viscera %	Viscera #	Limbs %	Limbs #	Struc-tural %	Struc-tural #	Toxic Response %	Toxic Response #	Functional %	Functional #
200.0	99	49.49	49	4.04	4	4.04	4	3.03	3					1.01	1						
160.0	99	50.50	50	1.01	1	2.02	2	1.01	1			1.01	1					1.01	1		
120.0	98	54.08	53	1.02	1	2.04	2	1.02	1			1.02	1								
80.0	134	51.49	69	2.23	3	2.23	3	2.23	3												
40.0	97	31.95	31	1.03	1	1.03	1					1.03	1								
0.0	107	48.59	52	0.00	0	0.00	0														
Prerced	60	38.33	23	0.00	0	0.00	0														
Untreated	238	7.98	19	0.42	1	0.00	0													0.42	1

SUMMARY - ALL DOSE LEVELS

527	47.82 252	1.90 10	2.28 12	1.52 8		0.57 3	0.19 1		0.19 1		

TABLE 4

ASCORBIC ACID
in WATER
YOLK - 96 HRS

Dose, ppm	No. Fertile	Mortality %	Mortality #	Abnormal Total %	Abnormal Total #	Abnormal H-S-V-L %	Abnormal H-S-V-L #	Abnormalities by category: Head %	Head #	Skeletal %	Skeletal #	Viscera %	Viscera #	Limbs %	Limbs #	Struc-tural %	Struc-tural #	Toxic Response %	Toxic Response #	Functiona %	Functiona #
200.0	118	24.57	29	0.00	0	0.00	0														
160.0	117	15.38	18	0.00	0	0.00	0														
120.0	120	18.33	22	0.83	1	0.83	1	0.83	1												
80.0	119	10.08	12	0.84	1	0.84	1	0.84	1												
40.0	119	16.80	20	0.00	0	0.00	0														
20.0	38	44.73	17	0.00	0	0.00	0														
0.0	198	13.13	26	2.02	4	1.51	3	1.01	2					0.50	1			0.50	1		
Pierced																					
ierced	98	19.38	19	1.02	1	1.02	1	1.02	1												
Untreated																					
reated	238	7.98	19	0.42	1	0.00	0													0.42	1

SUMMARY - ALL DOSE LEVELS

631	18.70 118	0.32 2	0.32 2	0.32 2							

TABLE 5

ASCORBIC ACID
in WATER
CHI-SQUARE ANALYSES of MORTALITY

Dose Level mg/kg	Air Cell		Yolk	
	0 hrs	96 hrs	0 hrs	96 hrs
4.0	4.91*(less)	0.00	-	-
12.0	6.06*(less)	0.07	-	-
20.0	2.93	0.67	-	19.30*
40.0	0.74	26.92*	5.17*(less)	0.54
80.0	0.12	124.07*	0.10	0.40
120.0	-	103.93*	0.42	1.20
160.0	-	103.93*	0.02	0.15
200.0	-	103.93*	0.00	5.96*
All Doses (DF)	12.37*(5)	355.15*(8)	12.46*(5)	31.40*(6)

* Probability $<$ 0.05 - 0.005.

TABLE 6

ASCORBIC ACID
in WATER
PROBIT ANALYSES - MORTALITY

	Air Cell		Yolk	
	0 hrs	96 hrs	0 hrs	96 hrs
LD-30 (Range)	NS	43.8 (36.0 - 50.4)	NS	NS
LD-50 (Range)	NS	57.7 (50.2 - 66.1)	NS	NS
LD-70 (Range)	NS	76.0 (66.3 - 91.7)	NS	NS
LD-90 (Range)	NS	113.1 (93.3 - 156.2)	NS	NS

TABLE 7

ASCORBIC ACID
in WATER
CHI-SQUARE ANALYSES of ABNORMALITIES

Dose Level mg/kg	Air Cell		Yolk	
	0 hrs	96 hrs	0 hrs	96 hrs
4.0	2.19	1.10	-	-
12.0	2.19	1.12	-	-
20.0	2.16	0.00	-	0.04
40.0	0.76	2.30	0.00	1.08
80.0	0.60	2.00	0.95	0.12
120.0	-	0.00	0.00	0.13
160.0	-	0.00	0.00	1.05
200.0	-	0.00	2.54	1.07
All Doses (DF)	10.86(5)	7.70(8)	6.55(5)	7.53(6)

TABLE 8

ASCORBIC ACID
in WATER
PROBIT ANALYSES - ABNORMALITIES

Air Cell		Yolk	
0 hrs	96 hrs	0 hrs	96 hrs
NS	NS	NS	NS

TABLE 9

ASCORBIC ACID
in WATER
CHI-SQUARE ANALYSES of HLSV ABNORMALITIES

Dose Level mg/kg	Air Cell		Yolk	
	0 hrs	96 hrs	0 hrs	96 hrs
4.0	1.49	0.00	-	-
12.0	1.49	1.12	-	-
20.0	1.47	0.00	-	0.00
40.0	0.32	0.51	0.00	0.56
80.0	0.83	0.84	0.95	0.00
120.0	-	0.00	0.00	0.00
160.0	-	0.00	0.00	0.54
200.0	-	0.00	2.54	0.55
All Doses (DF)	9.20(5)	7.19(8)	6.55(5)	5.34(6)

TABLE 10
ASCORBIC ACID in WATER
TERATOGENIC FINDINGS

TREATMENT	TOTAL NO. EXAMINED	TOTAL NO. ABNORMAL	SPECIFIC FINDINGS NO.	DESCRIPTION
ir Cell - 96 hrs 200.0 mg/kg	20	0	0	
160.0	20	0	0	
120.0	20	0	0	
80.0	119	3	1	dwarfism
			1	malrotation-hindlimb,unilateral; edema
			1	dwarfism; celosomia-abdomen
40.0	156	4	1	hypopigmentation-down
			1	dwarfism
			1	cachexia; abnormal curvature-hindlimb, unilateral
			1	dysgnathia-beak
20.0	100	0	0	
12.0	99	2	1	dysgnathia-beak
			1	anophthalmia-unilateral; dysgnathia-beak
4.0	100	2	1	agenesis-down
			1	dwarfism
0.0	157	0	0	

ASCORBIC ACID in WATER
TERATOGENIC FINDINGS

TREATMENT		TOTAL NO. EXAMINED	TOTAL NO. ABNORMAL	SPECIFIC FINDINGS NO.	DESCRIPTION
blk - 0 hrs	200.0 mg/kg	99	4	1	dysgnathia-beak
				1	anophthalmia-unilateral; dysgnathia-beak
				1	displacement-hindlimb, bilateral
				1	abnormal shortening-maxilla
				1	anophthalmia-bilateral; dysgnathia-beak; celosomia-abdomen; edema
	120.0	98	1	1	anophthalmia-bilateral; abnormal shortening-maxilla; celosomia-abdomen
	80.0	134	3	2	anophthalmia-unilateral; dysgnathia-beak
			1	1	anophthalmia-bilateral; abnormal shortening-maxilla
	40.0	97	1	1	fusion failure-abdomen
	0.0	107	0	0	
lk - 96 hrs	200.0	118	0	0	
	160.0	117	0	0	
	120.0	120	1	1	microphthalmia-unilateral
	80.0	119	1	1	anophthalmia-unilateral; dysgnathia-beak
	40.0	119	0	0	
	20.0	38	0	0	
	0.0	198	4	1	hypopigmentation-down
				1	abnormal curvature-hindlimb, unilateral
				1	exencephaly
				1	dysgnathia-beak